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Award Number: W81XWH-04-1-0264

TITLE: Identification of Markers of Human Vascular Dynamics Exposed in the Human Vasculature of Human Prostate Xenografts by Androgen Deprivation

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REPORT DATE: February 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	February 2005	Annual (23 Jan 04 - 22 Jan 05)

4. TITLE AND SUBTITLE
Identification of Markers of Human Vascular Dynamics Exposed in the Human Vasculature of Human Prostate Xenografts by Androgen Deprivation

5. FUNDING NUMBERS
W81XWH-04-1-0264

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The goals of this project are: 1) demonstration that androgen deprivation produces transient/permanent prostatic vascular damage; and, 2) characterization of vascular targets for molecular treatment modalities that are induced/unmasked by androgen deprivation. Progress during the initial year has focused on immunohistochemical evaluation of the kinetics of changes induced in the prostate vasculature and surrounding tissue, and on demonstration of induction of a pro-coagulative state indicative of acute vascular damage. Characterization of change in markers of vascular stability, and endothelial, epithelial and stromal cell proliferation and death, demonstrates clearly that the vascular endothelial compartment is labilized maximally at two days post-androgen deprivation, and rebounds between 7-14 days after androgen deprivation. Three-dimensional reconstruction of the prostatic vasculature from xenografts perfused with fluorescently labeled lectin demonstrates induction of areas of denuded vascular basement membrane is accompanied by leakage of lectin and fibrinogen into the interstitial tissue space and appearance of Tissue Factor on endothelial cell surfaces. Studies in year two will focus on verification of areas of vascular damage utilizing human platelets, and on characterization of new/unmasked targets by phage display. Induction of acute vascular damage suggests the opportunity for specific therapeutic targeting without risk of morbidity associated with long-term hormonal therapy.

14. SUBJECT TERMS
Prostate Vasculature; Phage Display, Castration; Androgen Deprivation; Human Prostate Xenografts

15. NUMBER OF PAGES
18

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION:

The objective of this project is to identify potential targets on human prostate vasculature that are altered/exposed in response to androgen withdrawal. These prostate specific targets can be exploited by chemo/molecular/immuno-therapeutic modalities to enhance death of prostate cancer (CaP) cells and/or prolong the clinical remission of CaP. Tumor vasculature, particularly in CaP, represents an important therapeutic target because: 1) vasculature in tumors is fundamentally different than the vasculature in the uninvolved organ; 2) endothelial cells of the prostate have the highest proliferative index of any organ (roughly 2.0% in the prostate compared to 0.01% in other organs); 3) over 40% of the vasculature in CaP is not associated with pericytes/smooth muscle cells (SMC) that stabilize the vessels, prevent proliferation/migration of the endothelial cells and stimulate basement membrane production; and 4) the vasculature of the prostate undergoes regression in response to androgen deprivation; The hypothesis of this proposal is that androgen withdrawal induces changes acutely in the prostate vasculature, heralded initially by rapid apoptotic death of endothelial cells, that will result in the presence of phenotypically altered endothelial cells and in the exposure of naked basement membrane, pericyte/smooth muscle cells and possibly the interstitial tissue space, including stromal and CaP cells. In Task 1, "Phage Display" will be utilized to identify human prostate vasculature specific markers common to prostate cancers from multiple patients, and to identify unique vascular markers exposed by the response of the human prostate tissue microenvironment to androgen deprivation. Immunocompromised host animals bearing human prostate xenografts will be infused intravenously with a commercially available phage display library, and phage that express peptides that bind specifically to targets on human prostate vasculature will be identified. In Aim 2, intact and castrate hosts bearing human prostate xenografts will be injected with fluorescently labeled lectins specific for endothelial cells, or basement membrane, and frozen sections imaged by confocal microscopy for 3-D reconstruction for visualization of the presence and pattern of denuded basement membrane. Immunohistochemistry (IHC) will be utilized to characterize the association of smooth muscle cells/pericytes with areas of intact versus denuded endothelium. Aim 3 will characterize the early expression of endothelial cell surface markers associated with cell activation/injury and the subsequent adhesion of host platelets, or exogenously added fresh or fixed human platelets, to denuded basement membrane induced by androgen deprivation of the host. These studies will focus on the targeting of platelet adhesion and the patterns of platelet adhesion/aggregation in the vasculature of the androgen deprived human prostate xenograft and the host (mouse) prostate.

BODY:

During the initial year of this project exciting progress was made towards addressing the goals of Tasks 2 and 3. These tasks described the experiments that would demonstrate the proof-of-concept for the project, that vascular damage was induced by androgen deprivation, and that there was a defined temporal pattern to the induction of vascular damage. During the next two years, the mechanistic studies in Task 1 will be pursued with the goals of demonstrating that areas of vascular damage are accessible to targeting, using human platelets as a prototypical targeting modality, and that unique targets are induced/unmasked in the human prostate vasculature in response to androgen deprivation, using the phage display technology. The exciting results developed during the initial year have resulted in an abstract to the annual American Association for Cancer Research meeting, a manuscript that is almost complete, and the establishment of a collaboration with HemoCellular Therapeutics that has led to the submission of an STTR Grant Application. HemoCellular Therapeutics has developed a technology for producing freeze-dried human platelets that they have been demonstrated to target areas of vascular damage. In addition, HemoCellular Therapeutics is developing mechanisms for loading prepared platelets with membrane markers and intracellular molecular moieties for specific delivery to areas of vascular damage. In that the corporate goal of HemoCellular Therapeutics is regulation of hemostasis at sites of vascular damage, expansion to delivery of toxicants to sites of vascular damage represents a mutually beneficial collaboration.

1. The approved studies described in Task 1 will *utilize the phage display technology to identify markers specific to the angiogenic vasculature of benign and malignant human prostate xenografts, and the acute changes in the marker profile elicited in response to androgen withdrawal*. The overall goal of these studies is to utilize "Phage Display" to identify human prostate vasculature specific markers common to benign prostate and prostate cancer from multiple patients, and to identify unique vascular markers exposed by the response of the human prostate vasculature (and tissue microenvironment) to androgen deprivation. As indicated above, the studies described in Task 1 that employ the phage display technology have not been implemented while the proof-of-concept studies described in Tasks 2 & 3 are being completed.

2. The approved studies in Task 2 will *characterize the induction of stretches of denuded vascular basement membrane, and the dynamics of smooth muscle cell/pericyte association/disassociation from the prostate neovasculature elicited in response to androgen withdrawal*. The overall goal of these studies is visualization and localization of areas of denuded vascular basement membrane by perfusion of xenografts maintained in androgen deprived hosts with fluorescently labeled lectins specific for endothelial cells, for vascular basement

membrane, or for both cells and basement membrane. In addition, immunohistochemistry (IHC) will be utilized to localize the multiple prostate cell types, characterize apoptosis/proliferation in the prostate cell populations, and to visualize differential association of smooth muscle cells/pericytes with vascular areas with intact and denuded endothelium.

a. Endothelial Cell Response to Androgen Deprivation in Human Xenografts

Primary xenografts of human prostate tissue were utilized to model changes in human prostatic vascular density, endothelial cell proliferation and apoptosis, and epithelial cell apoptosis in intact human prostate tissue *in vivo* in response to androgen deprivation. Xenografts were maintained on immunocompromised mouse hosts for one month after implantation for establishment and vascularization of the prostate tissue. In a pilot study of three patient samples, host animals with 30-day old xenografts were castrated, or mock castrated, and xenografts harvested at Day 0 (immediately after castration), and Day 2 and Day 7 after castration. Immunohistochemical analyses of the harvested xenograft material utilized a combination of the endothelial cell markers (CD31 & CD34) in conjunction with either a marker of apoptosis (activated Caspase-3) or a marker of proliferation (Ki-67). Histological analyses were quantitated as Mean Vessel Density (MVD), Vascular Endothelial Cell Proliferation Index (VPI), Vascular Endothelial Cell Apoptotic Index (VAI), and Glandular Epithelial Cell Apoptotic Index (EAI) (Fig. 1, A – F). There was a significant difference between the Mock and Castrate patient data set Area Under the Curve for both the MVD and VAI data sets ($p = 0.024$ and $p = 0.043$) (Fig. 1 A & B). Statistical comparison between two-day pairs revealed a statistically significant decrease in MVD between Day 0 and Day 2 ($p = 0.025$), as well as Day 0 and Day 7 ($p = 0.045$) in the xenografts from castrate animals. The VAI in castrates demonstrated an increase in apoptotic vessels, peaking at Day 2 and subsequently falling at day 7, while the mock castrates demonstrated a drop in vessel apoptosis at Day 2 that was maintained at Day 7. Based on the data in the pilot study that indicated a significant difference between xenografts in castrate and mock-castrate hosts, a large castrate only study was conducted using nine patient samples, with the xenografts harvested at five time points post-castration (Days 0, 1, 2, 4 and 7). This more complete study demonstrated the following trends: 1) The MVD decreased from Day 0 to Day 2, reaching a nadir, then increased to pre-castration levels by Day 4 (Fig. 1C); 2) The VPI is constant, but dips slightly on Day 2 (Fig. 1D); 3) The VAI increased significantly from Day 0 to Day 2 and decreased from Day 2 to Day 4-7 (Fig. 1E); and 4) The EAI increases steadily from Day 0 to Day 4, reaching a plateau (Fig. 1F). In summary, these data demonstrate that the maximal perturbation of vascular integrity occurs at Day 2 post-castration, with apoptosis in epithelial cells lagging several days behind the endothelial cells.

b. Expression of Androgen Receptor in Human Vascular Endothelial Cells:

Dual immunohistochemical staining for the combination of CD31/CD34 specific antibodies (FITC; green) and AR specific antibodies (Alexa Fluor 594; red) was performed to determine if there was AR expression by the prostate vascular endothelial cells. Testosterone-stabilized AR was identified sequestered in the nuclei of endothelial, epithelial and stromal cells in the human primary prostate xenograft model. In contrast, in saline perfused xenografts, or xenografts from castrate hosts, AR was localized in the cytoplasm. Analysis of co-localization by confocal laser scanning microscopy (CLSM) demonstrated a very high degree of co-localization (Pearson's correlation, Rr = 0.967815).

c. Changes in Cellular Signaling in Individual Prostate Cellular Compartments in Response to Androgen Deprivation:

Immunohistochemical staining protocols were developed to detect and localize Androgen Receptor (AR), Hypoxia Inducible Factor-1α (HIF-1 α), Vascular Endothelial Cell Growth Factor (VEGF), VEGF Receptor-2 (VEGFR–2), basic Fibroblast Growth Factor (bFGF), FGF Receptor-1 (FGFR-1), Platelet Derived Growth Factors (PDGF-AA & PDGF-BB), PDGF Receptor-α (PDGFR-α), Angiopoietin (Ang-1) and Ang-1 Receptor (Tie-2) in the prostatic endothelial, epithelial and stromal compartments. Trend analysis across the time points post-castration was performed using a 2-sided application of the Umbrella Test with Unknown Peak (Mack-Wolfe), tests for single peaks, single dips, steady increases or steady decreases. The test was applied to immunohistochemical stain scores by target protein by compartment, thereby making this a quantitative approach.

Endothelial cell levels of AR dropped by nearly one-half from Day 0 to Day 4, and rebound to nearly pre-castration levels from Day 4 to Day 7; the stromal cells demonstrated a nearly identical trend. In contrast, the AR levels in epithelial cells dropped slightly from Day 0 to Day 2, and remained level through Day 7 (Fig. 2A1-3). HIF-1α levels appeared to remain unchanged in all three prostate cell compartments across the time course after castration (Fig 2A1-3). Endothelial cell levels of VEGF increased nearly 6-fold from Day 0 to Day 2, and decreased to pre-castratel levels from Day 2 to Day 7 (Fig. 2C1). VEGF staining in stromal and epithelial staining intensities were very light (Fig. 2C2-3). Endothelial cell levels of VEGFR-2 increased gradually from Day 0 to Day 4, and decreased sharply from Day 4 to Day 7 (Fig 2D1-3). Basic-FGF levels increased in the endothelium from Day 0 to Day 2 and plateaued, while the epithelial and stroma levels demonstrated very light staining that did not change (Fig. 2E1-3). FGFR-1 expression levels were moderate to strong in all three compartments and did not respond to castration (Fig 2F1-3). PDGF-AA and PDGF-BB expression levels were low, and did not change in response to castration, in all three prostate cell compartments (Fig 3A1-3). PDGFR-A staining appeared to drop slightly at Day 2, and increase from Day 2 to Day 4, in all three cell compartments, however, the increase continued into Day 7 only in the endothelium and stroma (Fig 3C1-3). Ang-1 expression peaked in the endothelium on Day 2, however, the staining level was light and unchanged in the epithelium and stroma compartments (Fig. 3D1-3). Tie-2 levels increased 2-fold in the stroma from Day 0 to

Day 7, while the level of the receptor did not vary in endothelial cells and was undetectable in the epithelial compartment (Fig 3E1-3).

d. Visualization of Vascular Network and Areas of Denuded Vascular Basement Membrane:

The goal of these studies was to develop the technology to visualize the complex nature of the human vascular network that develops during establishment in the human prostate xenografts, the presence/lack of endothelial cells, and the induction of areas of denuded vascular basement membrane. Visualization of the vasculature of the prostate xenografts was accomplished by perfusion of the host with fluorescently conjugated lectin proteins and/or a combination of human specific antibodies for CD31 and CD34. Dual labeling of the vasculature of xenografts in castrated hosts with lectin and/or anti-CD31/34 antibodies supported strongly the induction of vascular damage in xenografts from castrated hosts because of the presence of lectin in the interstitial tissue space. Lectins were selected for visualization of the prostate vascular network based upon the significant literature. Type I lectins adhere to all areas of vasculature, whether coated with endothelial cell or having exposed basement membrane. In contrast, Type III lectins bind only to areas of denuded basement membrane. The cocktail of anti-CD31 and anti-CD34 antibodies was used to verify the presence of endothelial cells. Perfusion with the Type I lectin not only provided a clear "casting" of all of the vasculature in the xenografts, but in the 3-dimensional reconstructions of the xenografts produced from serial optical sectioning by CLSM demonstrated that in the androgen-deprived hosts there clearly was leakage of lectin into the interstitial tissue space that was absent in control xenografts (Fig 4a). A more definitive demonstration of vascular damage was provided in studies with perfusion of mice with Type I and Type III lectins. CLSM analysis of whole xenografts perfused simultaneously with both lectins demonstrated the presence of small areas marked by the Type III lectin (Fig 4b: red dye) in the vascular network (green dye) in xenografts harvested from a host two-days post-castration. The yellow color of stretches of vasculature indicates simultaneous binding of both lectins, indicating large stretches of damaged vasculature that can be recognized by both lectins. During year two we will develop the image processing technologies to allow quantitation of relative areas of intact endothelial surface and denuded vascular basement membrane.

3. The approved studies in Task 3 will *characterize early markers of human vascular damage and endothelial cell activation in response to androgen deprivation and the recognition of areas of denuded basement membrane by host and human platelets*. The overall goals of these studies are the characterization of the activation/injury of endothelial cells as indicated by the up-regulation of endothelial cell surface receptors associated with accumulation of inflammatory cells to sites of endothelial cell injury and by the expression of procoagulant Tissue Factor and fibrinogen in the human prostate xenografts and

host (mouse) prostate. Secondly, theses studies will characterize the adhesion of host platelets and exogenously added human fresh or fixed human platelets to the destabilized vasculature/denuded vascular basement membrane induced by androgen deprivation of the immunocompromised mouse host.

Primary xenografts of human prostate tissue were used to characterize the integrity of the prostatic endothelial cell lining, and the expression of the pro-coagulation factors Fibrinogen and Tissue Facto, in the prostate xenografts following castration of the immunocompromised mouse host. Vessels in the xenografts were identified with a cocktail of antibodies specific for human CD31 and CD34. Co-immunohistochemistry of the cocktail for human endothelial cells with anti-laminin antibodies allowed visualization of vessels with incomplete endothelial lining and exposed basement membrane (discussed above). Dual-label immunohistochemistry, using the CD31/CD34 vessel markers and either anti-fibrinogen or anti-Tissue Factor specific antibodies, demonstrated an androgen deprivation dependent were performed to identify induction of pro-thrombotic vessels with injured or activated endothelium. Dual-staining with the anti-endothelial marker and anti-fibrinogen demonstrated marked peri-vascular localization of fibrin deposits in castrated animals demonstrating increased vascular permeability (Fig 5A). Dual-staining with the anti-endothelial cell cocktail and anti-Tissue Factor antibodies demonstrated activation of Tissue Factor in the endothelial compartment of xenografts from castrate hosts, but not from mock castrate hosts (Fig 5B). This study confirms the observation discussed above of peri-vascular localization of perfused lectins, indicating that vascular leakage and pro-coagulability increase acutely following castration. Importantly, these studies increase the understanding of the timing of these physiologic changes in human prostate vasculature.

With the demonstration of acute vascular damage in response to androgen deprivation, studies characterizing the access of these sites to platelet adherence will be conducted early in year two of the project. HemoCellular Therapeutics, Inc. will provide fluorescently labeled, fixed human platelets that will be administered by perfusion either alone, or in conjunction with lectin/CD31-CD34 for visualization of the vascular network. Demonstration of specific binding of platelets to damaged vasculature in the prostate xenografts with minimal binding to the mouse host vasculature will suggest that platelets, or platelet-like artificial membrane bound vesicles could provide targeting vehicles for delivery of cytotoxic moieties to the prostate.

KEY RESEARCH ACCOMPLISHMENTS:

* Characterized the kinetics of perturbation of the human vasculature in the human prostate xenografts in response to androgen deprivation, identifying 2 days post-androgen deprivation as the time of greatest vascular perturbation.
* Demonstrated that human prostate endothelial cells express Androgen Receptor (AR) and undergo involution by apoptosis in response to androgen deprivation.
* Demonstrated that androgen deprivation creates acutely a pro-thrombotic environment in human prostate vasculature characterized by the induction of Tissue Factor on endothelial cells.
* Developed techniques to provide visualization of the entire vasculature network in the human xenografts by perfusion with fluorescently labeled lectin and imaging by confocal laser scanning microscopy (CLSM).
* Demonstrated the presence of areas of denuded vascular basement membrane by comparative staining with Group 1 lectins (adheres to endothelial cells and basement membrane) versus Group 3 lectins (adhere preferentially to areas of denuded basement membrane).
* Demonstrated leakage of serum components through the damaged vasculature, and into the interstitial tissue space, at times of maximal vascular perturbation as indicated by either leakage of Group 1 lectin into the interstitial tissue space or by the presence of fibrinogen in the interstitial tissue space.

REPORTABLE OUTCOMES:

1. Manuscript entitled "*Androgen Deprivation Induces A Biphasic Response in the Human Vasculature in Human Prostate Primary Xenografts: Rapid Involution of Endothelial Cells Reverses Rapidly in the Continued Absence of Androgen*" will be completed and submitted before the end of March 2005.

2. Abstract entitled "*Androgen ablation induced vascular labilization in the human prostate is associated with enhanced vascular permeability and enhanced coagulative state*" will be presented at the American Association for Cancer Research meeting in Anaheim, CA in March, 2005.

3. The work performed during this initial year, in conjunction with the new collaboration with HemoCellular Therapeutics, Inc. that was spawned by this DoD project, resulted in the submission of an STTR Grant Application in Nov. 2004 entitled "*Targeting Prostatic Revascularization in BPH and CaP*".

CONCLUSIONS:

Chemo/molecular/immuno-therapeutic modalities for the treatment of prostate cancer are ineffective, and androgen deprivation therapy, while capable of producing significant amelioration of symptoms contributing to morbidity, cannot prevent recurrence or reduce mortality from CaP. This project seeks to identify markers in the human prostate vasculature that may be present only transiently after initiation of androgen deprivation therapy, as potential targets for adjunct cytostatic/cytotoxic treatment modalities in CaP. The primary human prostate xenograft system provides a unique model for characterization of targets unmasked in the vasculature by androgen deprivation because xenografts from several patients can be established on a single host, facilitating identification of common markers of human prostate vasculature, or multiple xenografts from a single patient can be established on a single host, allowing characterization of the temporal response of the human vasculature to androgen deprivation. Experimental studies completed during the initial year of this project demonstrate clearly that androgen deprivation produces a marked, transient perturbation of the human prostatic vasculature in the human prostate xenografts demonstrated by apoptotic death of endothelial cells, appearance of areas of denuded vascular basement membrane, activation of markers of vascular damage including Tissue Factor and fibrinogen, and leakage of serum components into the interstitial tissue space. These studies validate the hypothesis of the project and demonstrate that the mechanistic studies to determine the availability of areas of damage vasculature to platelets, and to identify of unique markers produced or unmasked during vascular involution by phage display, are feasible and have a high likelihood of characterizing new therapeutic targets that are transiently available immediately after the inception of androgen deprivation therapy.

Prostate cancer is a disease unique among even other cancers. Current studies suggest that as many as 15% of twenty-year old men have histologically defined prostate cancer, and essentially all men over 70-75 have prostate cancer. However, the vast majority of these men die with CaP, not of Cap. Interpretation of this statistic in light of the lack of treatment of progressive CaP, including androgen deprivation therapy, indicates other therapeutic approaches must be considered. Identification of human prostate vasculature specific markers will allow design of molecular probes that can be conjugated with cytotoxic moieties to target for destruction only the human prostate. A realistic goal of such a therapeutic approach would be to block expansion of the CaP in situ, allowing natural death of the patient with the disease, not of the disease. Furthermore, transient treatments that interdict the androgen axis would have significantly less impact on quality-of-life issues than the morbidity that frequently accompanies long-term hormonal therapy.

APPENDICES:

1. Abstract to be presented at the AACR International Meeting in Anaheim, CA in March 2005.

Androgen ablation induced vascular labilization in the human prostate is associated with enhanced vascular permeability and enhanced coagulative state.

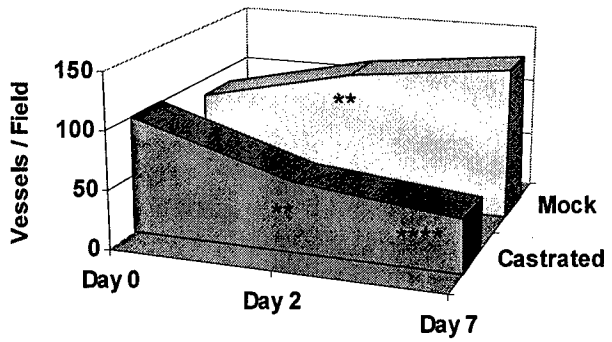
Danny R. Gray, Wendy J. Huss, Brian K. Buckley, Eric S. Werdin, Gary J. Smith. *University of North Carolina at Chapel Hill, Chapel Hill, NC.*

Androgen ablation causes prostatic endothelial cell death, vascular regression, and epithelial and stromal cell death. We hypothesized that physiologic changes to the androgen deprived vascular endothelium would increase labilization, total area of denuded basement and induce a coagulative state. A previously described model of vascular perturbation following castration in primary human xenografts of prostate tissue was used to characterize the integrity of the prostatic endothelial cell lining and the expression of the pro-coagulation factors Fibrinogen and Tissue Factor in the prostate following castration. Vessels in the xenografts were either perfused with fluorescently conjugated lectin proteins or identified with a combination of human specific antibodies specific for CD31 and CD34. Co-perfusion with anti-laminin antibodies allowed for dual labeling of areas of denuded basement membrane. Dual label immunohistochemistry with anti-fibrinogen or anti-Tissue Factor antibodies demonstrated an androgen deprivation dependent induction of pro-thrombotic vessels. In addition, peri-vascular localization of perfused lectins and fibrin deposits demonstrated increased permeability. This study confirms previous reports that vascular leakage and coagulability increase following castration and increases the understanding of the timing of these physiologic changes in human prostate vasculature. The presence of denuded basement membrane and endothelial cell expressed coagulation factors suggests that these findings may have clinical relevance.

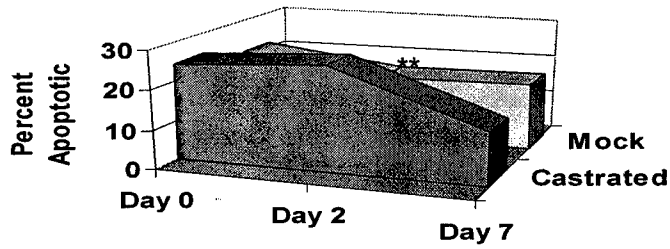
2. Figures Referenced in Body:

Figure 1.

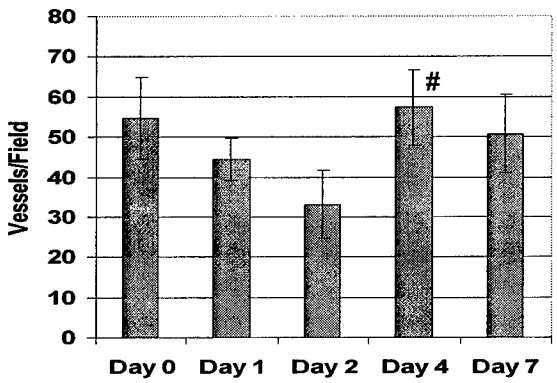
A) Post-Castration MVD: Mock vs. Castrate



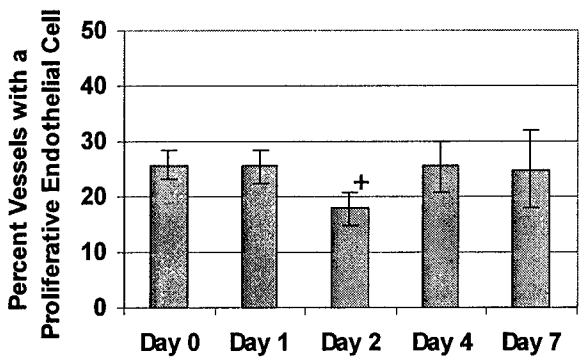
B) Post-Castration VAI: Mock vs. Castrate



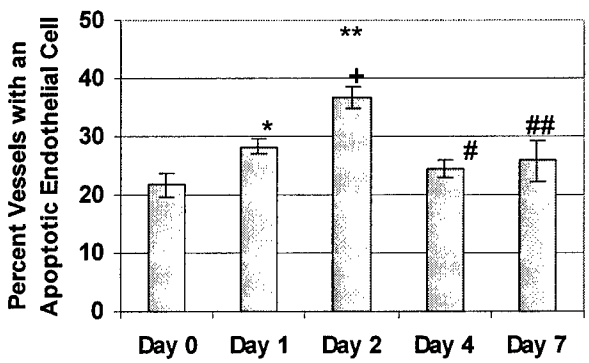
C) Effects of Castration on Mean Vessel Density



D) Castration Induced Endothelial Cell Proliferation



E) Castration Induced Endothelial Cell Apoptosis



F) Castration Induced Epithelial Cell Apoptosis

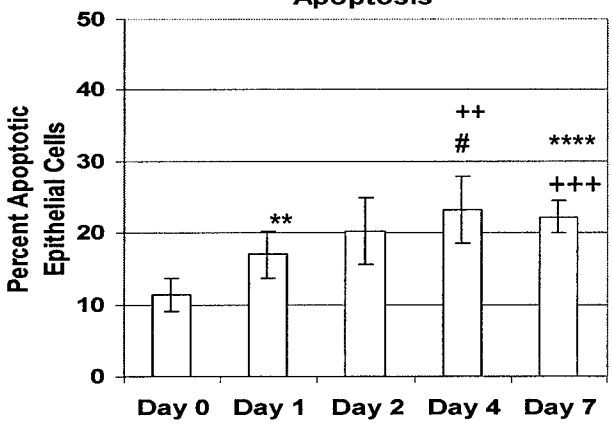
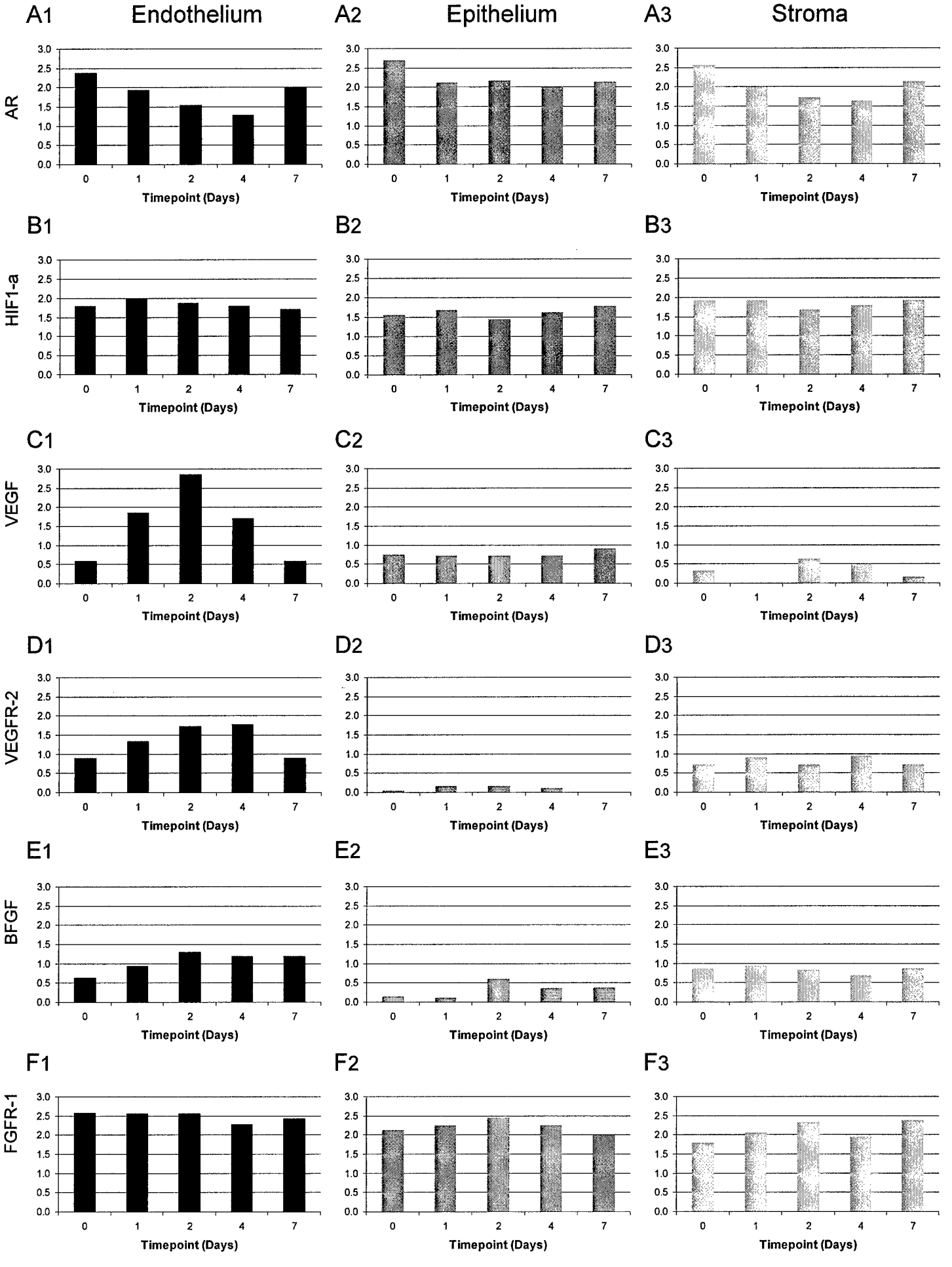


Figure 2.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Endothelium
A2
Epithelium
A3
Stroma
AR
B1
B2
B3
HIF1-a
C1
C2
C3
VEGF
D1
D2
D3
VEGFR-2
E1
E2
E3
BFGF
F1
F2
F3
FGFR-1
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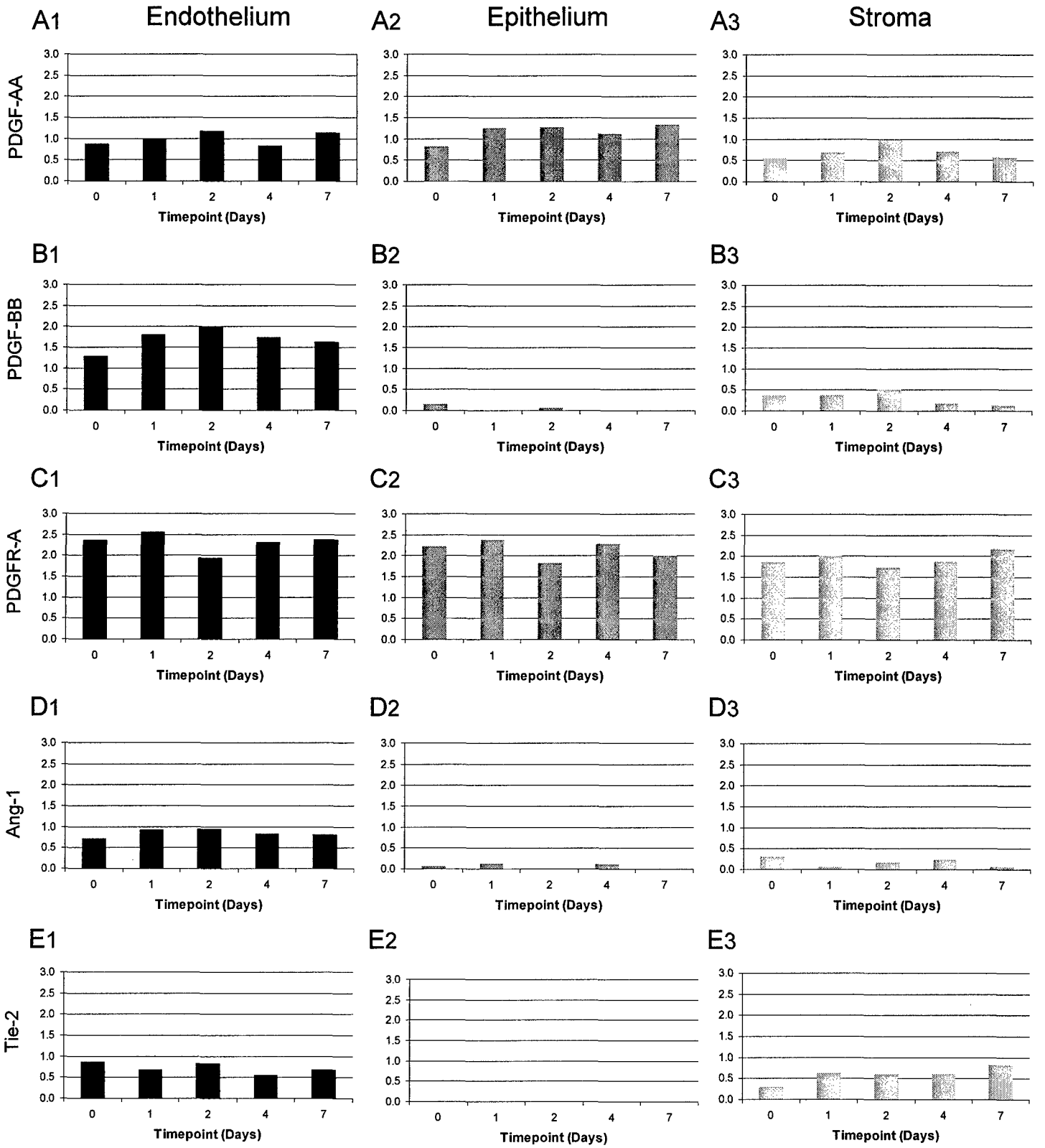
0
1
2
4
7
Timepoint (Days)

Figure 3.


A1
Endothelium
A2
Epithelium
A3
Stroma
PDGF-AA
B1
B2
B3
PDGF-BB
C1
C2
C3
PDGFR-A
D1
D2
D3
Ang-1
E1
E2
E3
Tie-2
3.0
2.5
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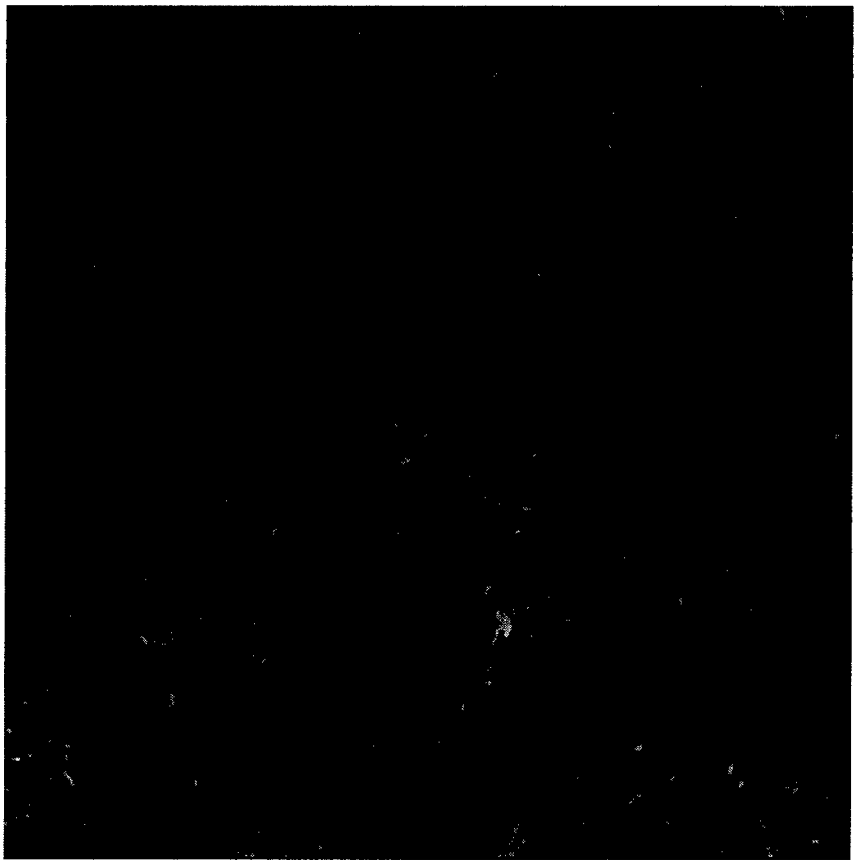
0
1
2
4
7
Timepoint (Days)

Figure 4.

a.



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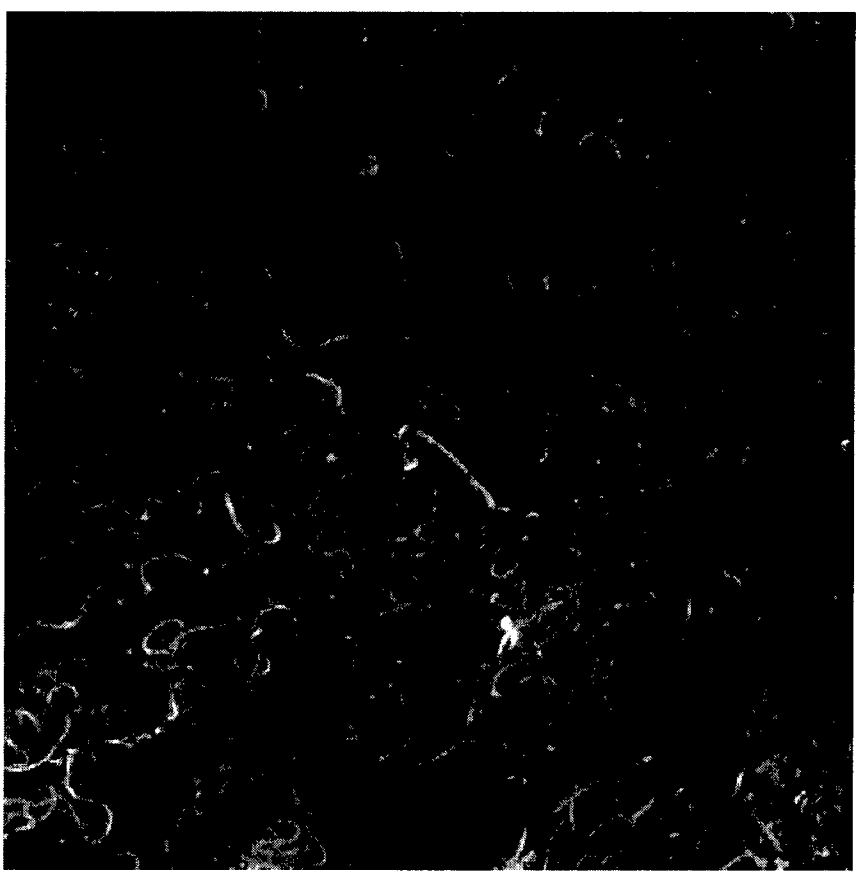
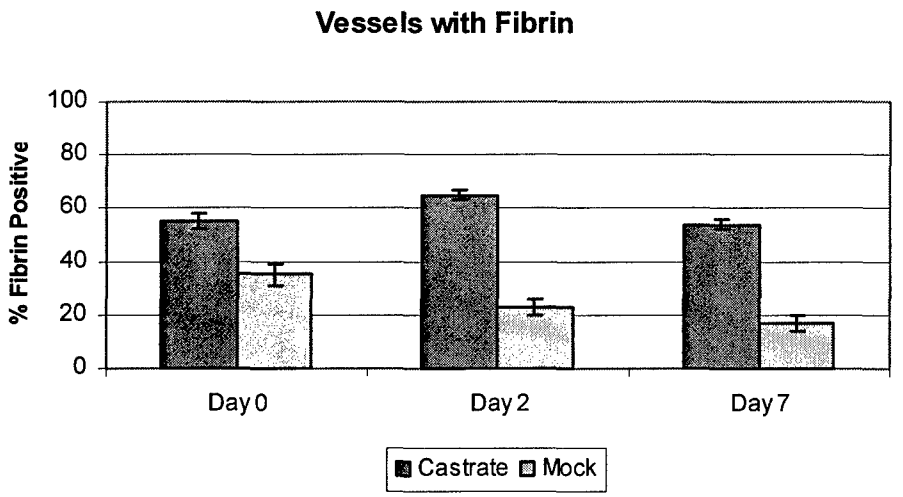


Figure 5.

A. Vessels with Fibrin Deposition



B. Tissue Factor Expression by Compartment